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(54) Title: A METHOD FOR THE MODULATION OF THE IMMUNE RESPONSE

(57) Abstract

A method of modulating or suppressing the immune response of an animal comprises the administration of an effective amount of at least one amphiphile which is capable of interacting at the surface of a cell to modify the surface properties thereof so as to inhibit or modify recognition of an antigen by the altered cell. A method of preparing animal tissue for grafting or transplantation from a donor animal to a recipient animal, and immunosuppressant compositions, are also disclosed. Preferably, the amphiphile is a cationic surfactant, such as a double-chained quarternary ammonium surfactant.

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A METHOD FOR THE MODULATION OF THE IMMUNE RESPONSE

This invention relates to a method of modulating the immune response in an animal (which term as used throughout this specification includes a human), and to compositions for use in the performance of such a method.

An animal's immune system is comprised of numerous elements that act separately and/or in concert to counteract, to eliminate, or to neutralise substances that are recognised by that system as foreign to the animal host. Generally, but not necessarily, the substance recognised as foreign by the immune system has its origin exogenous to the host. Exemplary of such exogenous substances are infectious bacteria and the by-products of their cellular activity, virus particles and their proteins, proteins injected by insect stings, and the like. In autoimmune diseases, such as rheumatoid arthritis, the host's immune system recognises host-made proteins or self-made proteins as foreign.

The immune response can be modified by artificial suppression (immunosuppression) or enhancement (immunopotentiation). Immunosuppression, i.e., artificially induced decreased responsiveness, can be achieved by six general methods: (1) administration of antigen, (2) administration of specific antisera or

antibody, (3) use of other biologic reagents such as antilymphocyte antisera, (4) use of drugs or hormones, (5) radiation, and (6) surgical removal of lymphoid tissue. Immunopotentiation can include the administration of an agent effecting an increase in the rate at which the immune response develops, an increase in the intensity or level of the response, a prolongation of the response, of the development of a response to an otherwise non-immunogenic substance. The agents which are known to enhance immune responses are generally termed adjuvants and can be placed into two general categories: (1) those providing general potentiation, i.e., substances which enhance both cellular and humoral immune responses for a wide variety of antigens, and (2) those providing specific potentiation, i.e., substances which enhance specific responses to certain antigens only.

To date, immunosuppressive drugs that have been developed to manipulate the immune response, are usually compounds of complex structure that have been discovered by accident. Further, their mode of action is often unknown or very unpredictable and administration of the drugs can be accompanied by undesirable side-effects.

It is a first object of the present invention to provide a method of modulating, and particularly of suppressing the immune response of an animal. A second object is to provide a method of modulating the immune response of an animal in which the active site of the substances used to modulate the immune response can readily be determined and easily altered to modify that immune response. A third object is to provide a method

of modulating the immune response without undesired side-effects. Other aims and objects of the present invention will become apparent from the following description.

There exists a class of compounds called "amphiphiles". The structure of these compounds comprises two dissimilar portions - a nonpolar, hydrophobic (repelled by water) "tail" portion which is lyophilic (attracted to fats) and often a hydrocarbon chain, and a polar, hydrophilic and lyophobic "head" portion. Many amphiphiles are commonly used as detergents. Detergents dissolve fatty materials and dirt by forming micelles, in which the oil or fat is at the centre of a sphere formed by the nonpolar hydrocarbon ends. The polar groups form a hydrophilic surface around the sphere and render the entire micelle water-soluble.

The present invention is based on the discovery that the nonpolar or tail end of certain amphiphiles can embed into the surface of a cell without necessarily destroying cell membrane function. The polar or head group of the amphiphile, now a part of the surface of the cell, modifies the local environment and thus alters the acceptor sites on the surface of that cell. In addition, local membrane curvature and thickness are perturbed, again modifying local microenvironment of cell attached peripheral molecules involved in immunorecognition.

According to the present invention, there is provided a method of modulating or suppressing the immune response of an animal, which comprises the

administration of an effective amount of at least one amphiphile which is capable of interacting at the surface of a cell to modify the surface properties thereof so as to inhibit or modify recognition of an antigen by the altered cell.

If desired, the amphiphile(s) may be administered in association with a pharmaceutically acceptable carrier or diluent.

In performing the method of this aspect of the invention, the amphiphile is administered at a concentration which is less than the concentration at which the cell membrane is disrupted and the cell lysed.

In another aspect of this invention, there is provided a composition suitable for modulation or suppression of the immune response of an animal, which composition comprises at least one amphiphile as described above together with a pharmaceutically acceptable carrier or diluent.

In yet another aspect, this invention relates to the use of at least one amphiphile as described above for the modulation or suppression of the immune response of an animal, and to the use of at least one amphiphile as described above for the production of a composition for use in the modulation or suppression of the immune response of an animal.

The present invention also extends to a method of preparing animal tissue for grafting or transplantation from a donor animal to a recipient animal, which method comprises the treatment of animal

tissue from said donor animal with an effective amount of at least one amphiphile as described above to inactivate cells in said tissue which stimulate the graft rejection reaction.

In particular, graft tissues removed from a donor animal, such as small cell grafts, (for example, endocrine tissues), may be exposed in vitro to the action of an amphiphile in accordance with this invention prior to being grafted into the recipient animal.

Amphiphiles, and particularly cationic surfactants closely related to those in widespread household and commercial use, have been shown to inhibit the generation of mixed lymphocyte reactions in vitro; to inhibit inflammatory responses caused by the injection of alloreactive cytotoxic cells into the footpads of mice; and to increase the susceptibility of mice to murine cytomegalovirus. This work confirms the potent immunosuppressive activity of these cationic surfactants.

Preferably, the amphiphiles used in the present invention are cationic surfactants of the general formulae:

$$R^1R^2X$$

wherein R^1 represents a straight- or branched-chain, saturated or unsaturated hydrocarbon of at least 8 carbon atoms;

R^2 represents a straight- or branched-chain, saturated or unsaturated hydrocarbon of at least 8 carbon atoms; and

X represents any suitable cationic moiety required to product an amphiphile; or

R^3X

wherein R^3 represents a straight- or branched chain, saturated or unsaturated hydrocarbon of at least 14 carbon atoms; and

X is as defined above.

In particularly preferred embodiments, R^1 and R^2 are chosen so that the total number of carbon atoms in the combined chains is 24 or fewer, or R^3 is chosen to have at least 16 carbon atoms in the chain. Typically, combinations of R^1 and R^2 include $C_{12}C_{12}$, C_8C_{12} and C_8C_{16}, whilst R^3 is typically C_{16}.

The present invention also extends to the use of mixtures of amphiphiles as broadly described above, including mixtures of double-chained amphiphiles of the formula R^1R^2X with single-chained amphiphiles of the formula R^3X.

It has been observed that the single- and double-chained cationic surfactants encompassed within the present invention exert their immunosuppressive effects by different mechanisms. Addition of single-chained surfactants to a cell-culture, or in vivo, will not destroy cell membranes, i.e. exhibit cell toxicity, and will exhibit immunosuppression below the critical micelle concentration (CMC), however they will be toxic above the CMC at which point spontaneous vesicles form and cell lysis occurs. Double-chained

surfactants on the other hand form vesicles rather than micelles and do not have a well-defined CMC, and it has been observed that such surfactants do not destroy cell membranes. Immunosuppression with these surfactants is not exhibited at longer chain lengths where the chain melting temperature is so high that aggregates are frozen lamellar phase inaccessible to cell membranes, i.e. the chain melting temperature of the surfactant should be in the range of 37-40°C or below for immunosuppression to be exhibited.

Particularly potent immunosuppressants, both in vivo and in vitro, are double-chained quaternary ammonium surfactants within this preferred class, more particularly didoecyldimethylammonium bromide (DDAB) and didodecyldimethylammonium acetate (DDAA).

The immunosuppressive activity of cationic surfactants has been tested by a study of the effect on the cell-mediated immune response, which provides a good model of cell/cell interactions. The results of this study, which are set out in detail below, have established that the immunosuppressive effect of these surfactants is not due to non-specific toxicity, but is probably due to impaired T-cell function.

The immunosuppressive activity of cationic surfactants is illustrated in the accompanying figures, in which:

Figure 1 shows lysis of P815 target cells by alloreactive B6 T_c cells generated in vitro in the presence of various concentrations of DDAB. (o) control; (●) 1 µg/ml; (△) 100 ng/ml; (▲) 10 ng/ml.

Figure 2 shows mortality of BALB/c female mice treated with 2 consecutive injections of 200µg DDAB i.v. (●) compared to saline treated controls (o), after challenge with 2 x 10^5 pfu MCMV i.p.

Figure 3 shows increase in footpad thickness of BALB/c female mice after injection of 3 x 10^6 T_c cells reactive against H-2^d alloantigens. Recipients were treated either with 2 consecutive injections of 200µg DDAB i.v. (Δ), or 2 mg in olive oil sc (▲). Controls (o) treated with saline or olive oil were not significantly different from each other, and were pooled for analysis. Each point represents the mean of a minimum of 5 mice. After 24 hr., footpad swelling in all mice treated with DDAB was significantly less than in the controls ($p < 0.05$). The vertical bars represent the standard deviations of the means.

Figure 4 shows lysis of P815 target cells by alloreactive B6 T_c cells generated in vitro in the presence of 10 ng/ml of DDAB (Δ) or DDAA (▲). Control (o).

Figure 5 shows lysis of P815 target cells by alloreactive B6 T_c cells generated in the presence of DDAA or cyclosporin A(CsA).

Figure 6 shows lysis of P815 target cells by alloreactive B6 T_c cells generated in the presence of various single- and double-chained cationic surfactants.

EXAMPLE 1

(i) Materials and Methods

Mice: BALB/c and C57 B1/6J (B6) mice were obtained from the Animal Breeding Establishment at the John Curtin School of Medical Research. Only female mice 6-12 weeks old were used in experiments.

Surfactants: Didodecyl dimethyl ammonium bromide (DDAB) and acetate (DDAA) were a gift from Dr. D.F.Evans, Department of Chemical Engineering, University of Minnesota. The former was purchased originally from Eastman Kodak and recrystallised twice from acetonitrile, the latter made by ion exchange to the hydroxide form in CO_2 free conditions, followed by neutralisation with acetic acid and lyphilisation. The bromide and acetate were used in saline suspension at 1 mg/ml; the bromide additionally in olive oil at 10 mg/ml. All preparations were sonicated to clear vesicular suspensions at 70 watts for two minutes before use.

Virus: Murine cytomegalovirus (MCMV) was grown in the salivary glands of 4-6 week old female BALB/c mice and stored at -70°C until used. Titres were determined by plaqueing on BALB/c mouse embryo fibroblasts.

Preparation of alloreactive Cytotoxic T (T_c) Cells: Cells reactive against BALB/c ($H\text{-}2^d$) alloantigens were obtained by culturing B6 spleen cells (2×10^6/ml) with irradiated (2000R) BALB/c spleen cells (5×10^5/ml) in Eagles Minimum Essential Medium supplemented with 5% foetal calf serum and 10^{-4} M 2-mercaptoethanol. In

certain experiments, appropriate concentrations of the surfactant were added at the initiation of the culture. At the conclusion of the 5 day culture period, the cells were harvested, resuspended in 0.5 ml of the medium, and lytic activity assayed on ^{51}Cr - labelled P815 ($H\text{-}2^{d}$) target cells. In order to determine the lysis per cell, a linear regression analysis was performed on the number of viable cells versus percent lysis at each dilution of the original culture. The results were then expressed as percent lysis at a specified effector-to-target cell ratio.

Graft-Versus-Host Response: Alloreactive T_c cells prepared as above were washed once, resuspended in Puck's balanced salt solution, and 3×10^6 cells in 40µl injected into the left footpads of BALB/c mice that had previously been treated with DDAB. An equal volume of Puck's saline alone was injected into the other footpad as a control. At various intervals thereafter, swelling was measured using a dial gauge caliper (H.C. Kroplin, Schluchtern, Hessen, FGR). The results were expressed as the difference in thickness between left and right footpads.

(ii) Results

Initial experiments were designed to investigate the effect of DDAB on alloreactive T_c cells, either generated or assayed in the presence of the surfactant. Fig.1 shows that there was substantial loss of effector cell activity in the cultures, even at concentrations as low as 10 nanograms/ml. However, because DDAB was toxic to lymphocytes when used at 1µ/ml, the yields of cells from the cultures were reduced at lower concentrations

of the surfactant, it was essential to determine whether the apparent immunosuppressive effect was simply due to the presence of proportionally fewer effector cells. This possibility can be dismissed. When the data were corrected for lysis on a cell-for-cell basis (Table 1) it was clear that effector cell function had indeed been impaired. In contrast, when the T_c cell activity of control cultures was assayed in the presence of similar concentrations of DDAB, there was no loss of effector cell function (data not shown).

TABLE 1

Corrected lysis of P815 target cells by alloreactive B6 cells generated in the presence of various concentrations of didodecyl dimethyl ammonium bromide

Treatment	Percent lysis[1]
None	74.3
1µg/ml	0[2]
100µg/ml	36.8
10ng/ml	33.0

[1] Calculated from linear regressions, at a killer-to-target ratio of 2.5:1

[2] No viable cells were recovered from the culture.

Given the above, it was important to establish whether the result could be attributed to some singularity of the tissue culture system, or whether the surfactant also had some biological activity in vivo. In order to discriminate between these alternatives, BALB/c mice treated with two consecutive injections of

DDAB i.v. were challenged with a minimum lethal dose (2 x 10^5 pfu) of MCMV. These mice showed a sharp reduction in median survival time compared to infected mice pretreated with saline (Fig.2). Because anti-viral T_c cells are essential for recovery from primary viral infection, an increased susceptibility to MCMV infection was consistent with the impaired generation of T_c cell function obsereved in vitro. A further experiment was devised to examine the function of alloreactive T cells, generated in vitro, after transfer into DDAB treated mice. The reactivity of these cells, as measured by the increase in footpad swelling, was substantially reduced (Fig.3). These in vivo phenomena depend not only on effector cell function, but also on movement of T lymphocytes and other cell types from the blood to the site of antigenic challenge. As cell recirculation patterns could also be disrupted by the presence of a surfactant, the precise mechanism(s) of action must await further analysis.

Attempts to determine whether intranasal administration of DDAB would also cause generalised immunosuppression were unsuccessful because at the dose used (50µg) the surfactant apparently interfered directly with lung function, and the mice died within 24 hr.

The marked immunosuppressive potential exhibited by DDAB both in vitro and in vivo is manifest. It was of interest to enquire further if the mode of delivery of surfactant vesicles altered these biological effects. Sonicated DDAB suspensions form small (diameter = 500 A) cationic vesicles admixed with liquid crystallites which in the presence of saline further flocculate to increase

the proportion of less accessible liquid crystals. By contrast, using acetates or other carboxylates as counterions, vesicles and micelles form spontaneously in aqueous solution, and in saline revert to liquid crystals much more slowly. The expectation was then that this circumstance would result in a freer passage of surfactant molecules into the cell membrane by heterocoagulation. The relative effects of the two salts on the generation of alloreactive cells in vitro is shown in Fig.4 and the results are in accord with prediction. T_c cells cultured in the presence of DDAA showed less effector activity than those grown in the presence of the bromide and this difference was even more apparent when adjustment was made to compensate for different cell yields (Table 2). Vesicles made by polymerising the counterions should be even more effective, and have the virtue of enabling simultaneous delivery of encapsulated additional agents.

TABLE 2

Corrected lysis of P815 target cells by alloreactive B6 cells generated in the presence of didodecyl dimethyl ammonium bromide or acetate

Treatment[1]	Percent lysis[2]
None	45.9
DDAB	38.1
DDAA	18.2

[1] Surfactants were added to the culture at a concentration of 10ng/ml.

[2] Calculated from linear regressions, at a killer-to-target cell ratio of 5:1.

EXAMPLE 2

The potency of the immunosuppressive effects of DDAA have been compared with those of cyclosporin A (CsA) dissolved in dimethylsulphoxide. The materials and methods used were based on those described for Example 1 above. Figure 5 shows lysis of P815 target cells by alloreactive B6 T_c cells generated in the presence of 1 µg/ml CSA (•) 100 ng/ml CsA (Δ), 10 ng/ml CsA (□), 1 µg/ml DDAA ■, 100 ng/ml DDAA (◇), and 10 ng/ml DDAA (▽), Control (o).

A surfactant with a single chain $C_{16}Br$ and surfactants with chains of unequal length, such as $C_8C_{12}Br$ and $C_8C_{16}Br$ partly suppress T lymphocyte responses <u>in vitro</u> whereas another single chained surfactant $C_{12}Br$ and other double-chained surfactants with slightly longer chains such as $C_{12}C_{18}Ac$ or $(C_{16})_2Ac$ are relatively inactive. Figure 6 shows lysis of P815 target cells by alloreactive B6 T_c cells generated in the presence of 100ng/ml $C_{16}Br$ (□), 100ng/ml $C_8C_{12}Br$ (Δ), 100ng/ml $C_{12}Br$ (■), 200ng/ml $C_{12}C_{18}Ac$ (•) and 200ng/ml $(C_{16})_2Ac$ (▽), Control (o).

CLAIMS

1. A method of modulating or suppressing the immune response of an animal, which comprises the administration of an effective amount of at least one amphiphile which is capable of interacting at the surface of a cell to modify the surface properties thereof so as to inhibit or modify recognition of an antigen by the altered cell.

2. A method according to claim 1 wherein the amphiphile is administered in association with a pharmaceutically acceptable carrier or diluent.

3. A method according to claim 1 or claim 2 wherein the amphiphile is administered at a concentration which is less than the concentration at whhich the cell membrane is disrupted and the cell lysed.

4. A composition suitable for modulation or suppression of the immune response of an animal, which composition comprises at least one amphiphile which is capable of interacting at the surface of a cell to modify the surface properties thereof so as to inhibit or modify recognition of an antigen by the altered cell, together with a pharmaceutically acceptable carrier or diluent.

5. The use of at least one amphiphile which is capable of interacting at the surface of a cell to modify the surface properties thereof so as to inhibit or modify recognition of an antigen by the altered cell,

for the modulation or suppression of the immune response of an animal.

6. The use of at least one amphiphile which is capable of interacting at the surface of a cell to modify the surface properties thereof so as to inhibit or modify recognition of an antigen by the altered cell, for the production of a composition for use in the modulation or suppression of the immune response of an animal.

7. A method of preparing animal tissue for grafting or transplantation from a donor animal to a recipient animal, which method comprises the treatment of animal tissue from said donor animal with an effective amount of at least one amphiphile which is capable of interacting at the surface of a cell to modify the surface properties thereof so as to inhibit or modify recognition of an antigen by the altered cell, to inactivate cells in said tissue which stimulate the graft rejection reaction.

8. A method, composition or use according to any one of claims 1 to 7 wherein the amphiphile is a compound of the general formula

$$R^1R^2X$$

wherein

R^1 represents a straight- or branched-chain, saturated or unsaturated hydrocarbon of at least 8 carbon atoms; R^2 represents a straight- or branched-chain, saturated or unsaturated hydrocarbon of at least 8 carbon atoms; and X

represents any suitable cationic moiety required to produce an amphiphile; or

$$R^3X$$

wherein

R^3 represents a straight- or branched-chain, saturated or unsaturated hydrocarbon of at least 14 carbon atoms; and X is as defined above.

9. A method, compositon or use according to claim 8 wherein R^1 and R^2 are chosen so that the total number of carbon atoms in the combined chains is 24 or fewer, or wherein R^3 is chosen to have at least 16 carbon atoms in the chain.

10. A method, composition or use according to any one of claims 1 to 9 wherein said amphiphile is a double-chained quarternary ammonium surfactant.

11. A method, compositon or use according to claim 10 wherein said double-chained quarternary ammonium surfactant is didodecyldimethylammonium bromide, or didodecyldimethylammonium acetate.

FIG.1

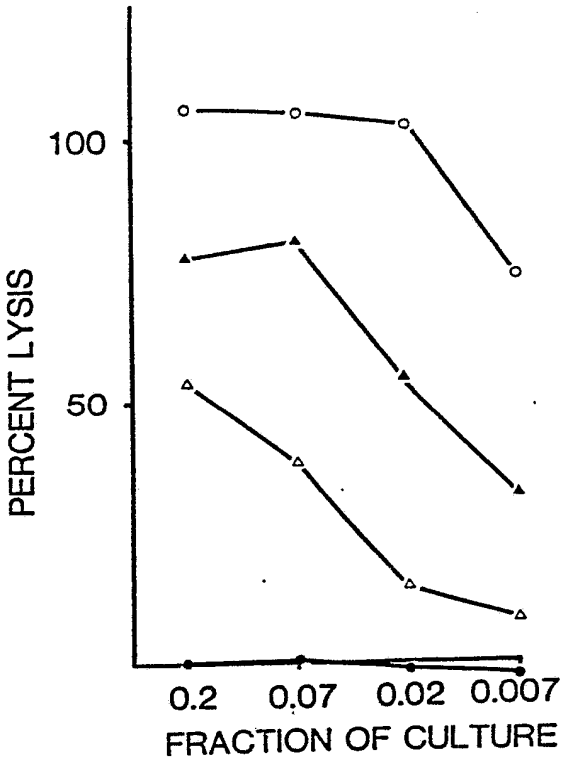

100
50
PERCENT LYSIS
0.2
0.07
0.02
0.007
FRACTION OF CULTURE

FIG.2

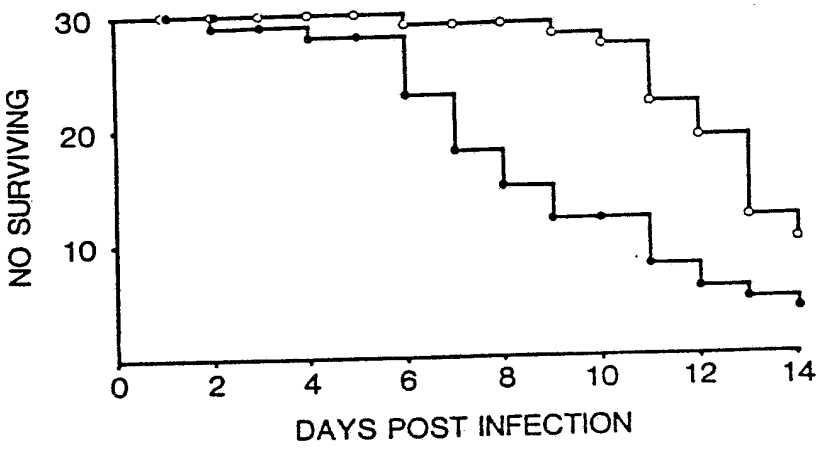

30
20
10
NO SURVIVING
0
2
4
6
8
10
12
14
DAYS POST INFECTION

FIG.3

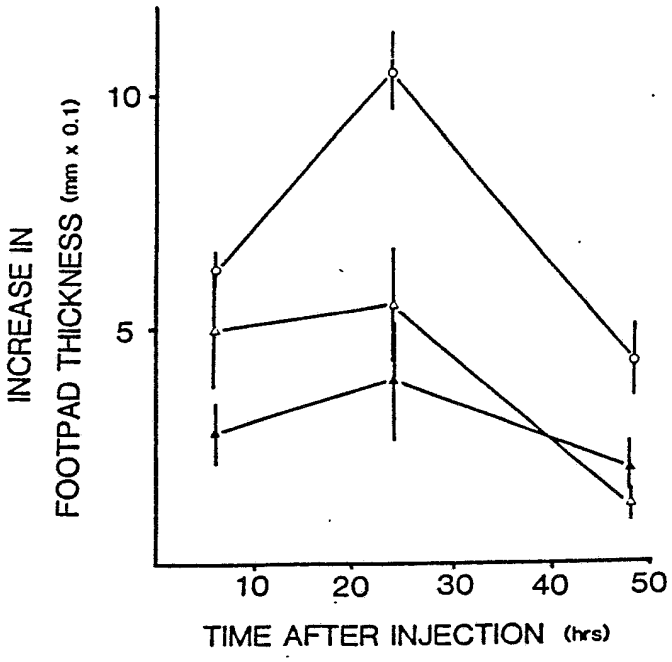

INCREASE IN
FOOTPAD THICKNESS (mm x 0.1)
10
5
10
20
30
40
50
TIME AFTER INJECTION (hrs)

FIG.4

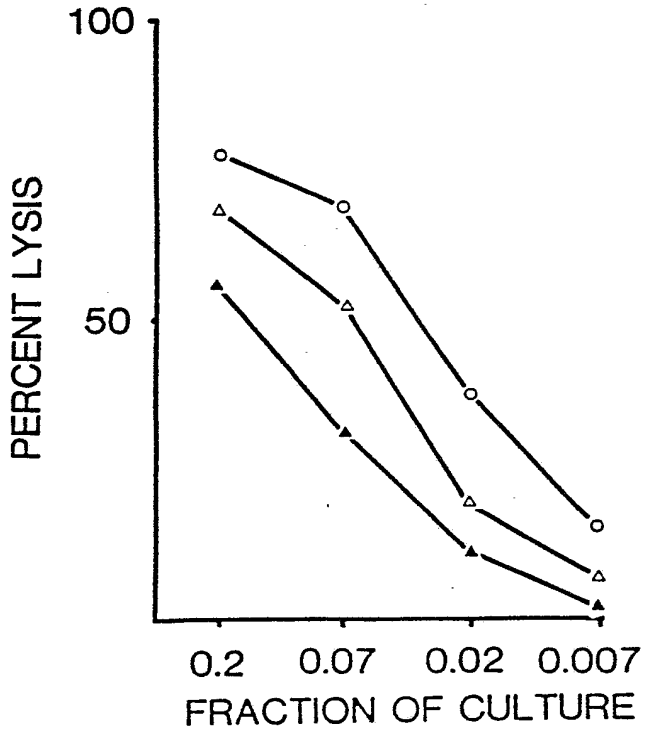
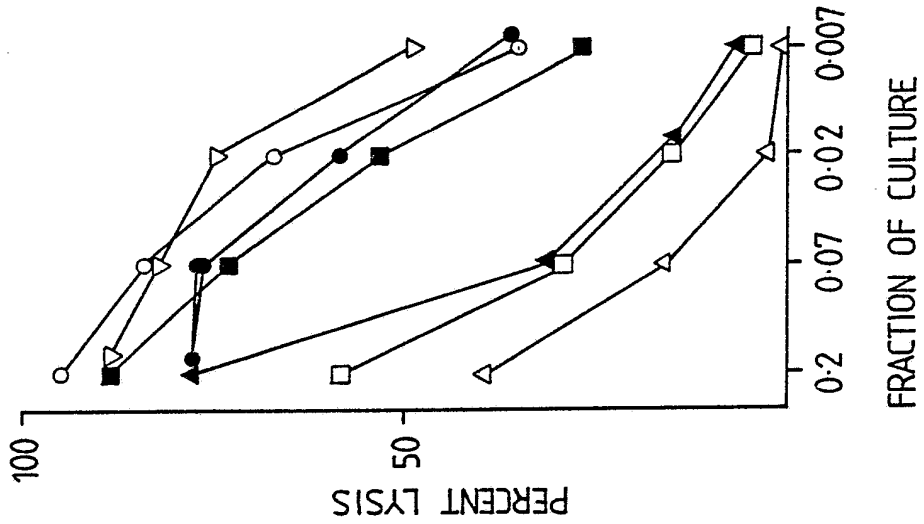

100
50
PERCENT LYSIS
0.2
0.07
0.02
0.007
FRACTION OF CULTURE

FIG 6


100
50
PERCENT LYSIS
0·2
0·07
0·02
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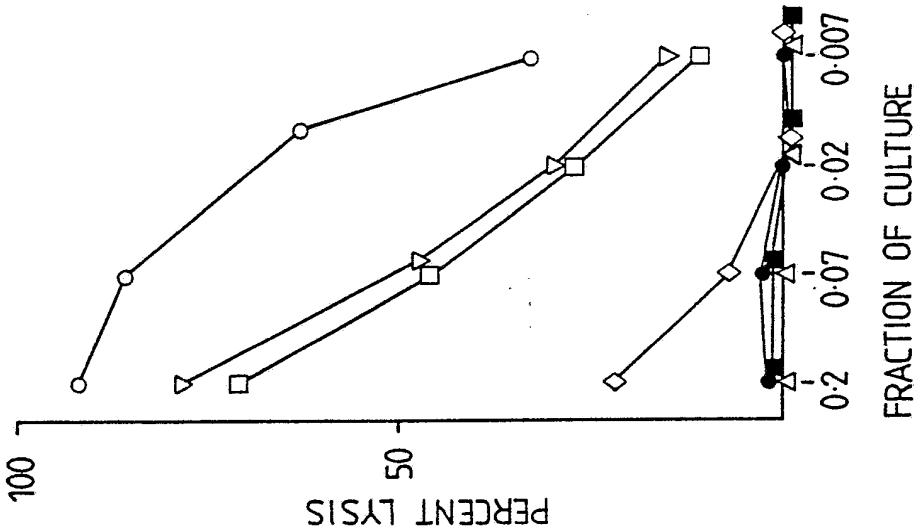
FRACTION OF CULTURE

FIG 5


100
50
PERCENT LYSIS
0·2
0·07
0·02
0·007
FRACTION OF CULTURE

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 85/00101

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] A61K 31/14, C12N 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	A61K 31/14, C11D 1/62, C12N 5/00, 5/02
US Cl.	424/329

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU: IPC as above; Australian Classification 87.16-0

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	AU,B, 18649/56 (222828) (IMPERIAL CHEMICAL INDUSTRIES OF AUSTRALIA AND NEW ZEALAND LIMITED) 21 November 1957 (21.11.57) See page 1.	(4,8)
X	AU,B, 33748/68 (417584) (BAIRD CHEMICAL INDUSTRIES, INC.) 21 August 1969 (21.08.69) See page 3.	(4)
X	AU,A, 38253/68 (DERMAL LABORATORIES, LTD) 27 November 1969 (27.11.69) See page 3.	(4)
X	AU,B, 54773/73 (476608) (ENVIRONMENTAL SCIENCE CORPORATION) 24 October 1974 (24.10.74) See pages 16 and 21.	(4,8)
X	US,A, 2653156 (MARTIN et al) 22 September 1953 (22.09.53) See column 1 lines 6-35.	(4)
X	US,A, 3950541 (WALDSTEIN) 13 April 1976 (13.04.76)	(4)
X	US,A, 4320147 (SCHAEUFELE) 16 March 1982 (16.03.82) See column 1 lines 25-35.	(4,8)
X	US,A, 4330551 (STOUT et al) 18 May 1982 (18.05.82) See column 2 lines 21-30.	
X,P	US,A, 4450174 (GREEN et al) 22 May 1984 (22.05.84) See column 1 lines 5-19.	(4,8)

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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
4 July 1985 (04.07.85)	(12.07.85) 12 JULY 1985
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	J.W. ASHMAN

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Japanese Journal of Medical Science & Biology, Volume 31, No. 4, issued 1978 August (Tokyo, Japan), J. Chiba and Y. Egashira, 'Adjuvant Effect of Cationic Surface-active Lipid, Dimethyl Dioctadecyl Ammonium Bromide, on the Induction of Delayed-Type Hypersensitivity to Sheep Red Blood Cells in Mice'.	(4,5)

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 1-3, because they relate to subject matter not required to be searched by this Authority, namely:

Method for treatment of the human or animal body by surgery or therapy.

2. [ ] Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU **85/00101**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
AU 33748/68	BE 701166	BE 710970	BE 710971
	CH 501580	CH 2588/68	CH 526906
	DE 1668871	DE 1643235	ES 350826
	ES 350827	FR 1572247	FR 1572248
	GB 1221224	LU 54078	LU 55555
	LU 55557	NL 6709647	NL 6802352
	NL 6802353	US 3836669	
AU 38253/68	FR 1595553	GB 1155258	IL 30063
AU 54773/73	BE 798759	CA 1012463	CH 575759
	DE 2321596	FR 2183022	GB 1434757
	JP 49041516	NL 7305886	US 3869550
	ZA 732782		
US 4330551	CA 1179604	EP 46594	JP 57118514

END OF ANNEX

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 85/00099

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
AU 68728/81	AR 226718	BR 8101783	CA 1174932
	CA 1179231	DK 1351/81	EP 40683
	ES 500704	FI 810916	JP 57009444
	MX 150766	NO 811012	US 4406392
	ZA 812018		
AU 85692/82	CA 1181646	EP 69557	JP 58019242
	ZA 824831		
AU 11279/83	DE 3204532	EP 85930	JP 58138447
	US 4505273		
AU 81476/82	CA 1187762	EP 70307	US 4375866
	WO 8202486		
AU 72709/81	BR 8108844	CA 1170537	EP 50554
	GB 2098696	US 4489875	WO 8201308

END OF ANNEX